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A Novel *cdsAB* Operon Is Involved in the Uptake of L-Cysteine and Participates in the Pathogenesis of *Yersinia ruckeri*▽

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Application of *in vivo* expression technology (IVET) to *Yersinia ruckeri*, an important fish pathogen, allowed the identification of two adjacent genes that represent a novel bacterial system involved in the uptake and degradation of L-cysteine. Analysis of the translational products of both genes showed permease domains (open reading frame 1 [ORF1]) and amino acid position identities (ORF2) with the L-cysteine desulfidase from *Methanocaldococcus jannaschii*, a new type of enzyme involved in the breakdown of L-cysteine. The operon was named *cdsAB* (cysteine desulfidase) and is found widely in anaerobic and facultative bacteria. *cdsAB* promoter analysis using *lacZY* gene fusion showed highest induction in the presence of L-cysteine. Two *cdsA* and *cdsB* mutant strains were generated. The limited toxic effect and the low utilization of L-cysteine observed in the *cdsA* mutant, together with radiolabeled experiments, strongly suggested that CdsA is an L-cysteine permease. Fifty percent lethal dose (LD_{50}) and competence index experiments showed that both the *cdsA* and *cdsB* loci were involved in the pathogenesis of the bacteria. In conclusion, this study has shown for the first time in bacteria the existence of an L-cysteine uptake system that together with an additional L-cysteine desulfidase-encoding gene constitutes a novel operon involved in bacterial virulence.

The genus *Yersinia* is known mainly because it includes three important human pathogenic species, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*, which have been deeply studied in the last 20 years. However, the genus also includes other species, such as *Yersinia ruckeri*, involved in pathological processes in intensive aquaculture which cause important economic losses in this kind of industry. This bacterium is the etiological agent of the enteric red mouth disease of fish. This disease is spread throughout the world and affects mainly the aquaculture of salmonids. Most of the mechanisms involved in the virulence of *Yersinia* species that cause human diseases are very well known. In contrast, only a few pathogenic mechanisms of *Y. ruckeri* have been described (7, 12, 14, 29). The application to *Y. ruckeri* of *in vivo* expression technology (IVET) has allowed the identification of 14 genes specifically induced during the infection process in rainbow trout (11). Some of them have been proven to participate in virulence, such as the iron uptake mechanism via the catecholate siderophore ruckerbactin (11), the YhlA hemolysin (13), and the *tra* chromosomally located operon, a type IV secretion system (22).

Cysteine is an important amino acid, because as a sulfur-containing compound, it is the sole entrance for reduced sulfur into cell metabolism in most organisms. Cysteine is essential for the biogenesis of Fe-S clusters of some enzymes; it also plays a crucial role in protein folding through disulfide bond formation, and the sulfur component is needed for synthesis of essential compounds, such as methionine, thiamine, biotin, etc. This amino acid residue also represents the rate-limiting nutrient in glutathione biosynthesis (1, 34), the major redox buffer and detoxification molecule in the cell. In spite of these important roles of cysteine, an increased cysteine level has been shown to be toxic to cells (18, 19, 26). For this reason, the intracellular cysteine levels need to be tightly regulated, by controlling not only its biosynthesis and degradation but also the transport of this amino acid from the extracellular medium to the cell and vice versa. In contrast to eukaryotes, cysteine uptake has been poorly characterized in bacteria (10, 24, 32). These systems are rare in prokaryotes, in part because this amino acid is readily oxidized to the disulfide-linked cystine and taken up in this form. For this reason, cystine uptake has been further studied (5, 6, 17).

There are several types of cysteine-degrading enzymes in bacteria. These include L-cysteine desulfurase (EC 2.8.1.7) and D-cysteine desulfhydrase (EC 4.4.1.15), both pyridoxal phosphate-dependent enzymes. Recently, a new type of L-cysteine-degrading enzyme has been described in the archaeal *Methanocaldococcus jannaschii* (33). This was defined as L-cysteine desulfidase and uses the [4Fe-4S] center instead of pyridoxal phosphate to catalyze the hydrolysis of L-cysteine to sulfide, ammonia, and pyruvate. The enzyme was oxygen sensitive, and on the basis of sequence comparison it was found that this protein was widely present in anaerobic bacteria (33).

In this paper, the analysis of a previously selected *iviX* clone (11) showed the presence in *Y. ruckeri* of a novel two-component operon involved in the assimilation of L-cysteine. According to sequence analysis, the first gene corresponds to an amino acid permease and the second to an L-cysteine desulfidase. Regulation studies and uptake experiments confirm the involvement of this system in the cysteine metabolism of the bacterium. Interestingly, the operon is needed for full virulence of *Y. ruckeri* in fish.

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Relevant properties	Source or reference
Strains		
Yersinia ruckeri		
150R	Rif^r derivative of strain 150	14
150R*iviX*	Strain containing *ivi* fusion expressed only in the host	11
cdsA mutant	Rif^r *cdsA*::pJP5603	This work
cdsB mutant	Rif^r *cdsB*::pJP5603	This work
146, 147, 148, 149, and 150	Strains isolated from an outbreak in trout (Danish fish farm)	J. L. Larsen (Denmark)
955, 956, and 43/19	Strains isolated from an outbreak in trout (U.S. fish farms)	CECT (Spanish Type Culture Collection)
35/85 and 13/86	Strains isolated from outbreaks in trout (Danish and United Kingdom fish farms, respectively)	C. J. Rodgers, University of Tarragona, Spain
A100 and A102	Strains isolated from outbreak in trout (Spanish fish farm)	I. Márquez, Laboratory of Animal Health
150/05, 158/05, and 382/05	Strains isolated from outbreak in trout (Spanish fish farm)	Proaqua Nutrición S.A.
Escherichia coli		
S17-1λ*pir*	Sm^r λ(*pir*) *hsdR pro thi* RP4-2 Tc::Mu Km::Tn*7*	31
MT1694	Helper strain HB101 containing pRK2013	15
Plasmids		
pIVET8	Ap^r *ori*R6K *mob*$^+$ promotorless *cat-lacZY*	20
pJP5603	Kan^r *ori*R6K *mob*$^+$ P*lac* promoter	27
pCdsA	pJP5603::BamHI (*cdsA*), Kan^r	This work
pCdsB	pJP5603::BamHI (*cdsB*), Kan^r	This work

[a] All *Y. ruckeri* strains with the exception of strain 956 (which belongs to serotype 2) belong to serotype 1. Rif^r, rifampin resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Kan^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Y. ruckeri* strains were routinely cultured in nutrient broth (NB; Difco) and on nutrient agar (NB with 1.5% agar) (NA) and M9 medium supplemented with 0.5% glucose (Scharlau Chemie S.A.) and 0.2% Casamino Acids (Becton, Dickinson and Company) (M9GC) at 18°C or 28°C. *Escherichia coli* strains were cultured in 2× tryptone-yeast extract (TY) broth or agar. In order to avoid L-cysteine oxidation, the bacteria were incubated in 50-ml Falcon screw-top airtight tubes containing 20 ml of medium. Under this condition, L-cysteine was stable as determined by the ninhydrin quantification method (16) (see below).

For incubation under aerobic conditions, 250-ml Erlenmeyer flasks containing 20 ml of medium were inoculated with the bacteria and incubated in an orbital shaker at 250 rpm. If required, the following antibiotics were added to the medium: 100 μg/ml ampicillin, 50 μg/ml rifampin, 50 μg/ml streptomycin, and 50 μg/ml kanamycin, all of them from Sigma-Aldrich Co. Growth was monitored by determining the optical density at 600 nm (OD_{600}) of a culture with a Hitachi U-2900 spectrophotometer at different times during incubation at the appropriate temperature.

Genetic techniques. Routine DNA manipulation was performed as described by Sambrook and Russell (30). Phage T4 DNA ligase and calf intestinal alkaline phosphatase were purchased from Roche Ltd., restriction enzymes were from Takara Bio Inc., and oligonucleotides were from Sigma-Aldrich Co.

DNA sequencing was performed by the dideoxy chain termination method with the BigDye Terminator version 3.1 (Applied Biosystems) according to the manufacturer's instructions in an ABI Prism 3130xl DNA sequencer at the Universidad de Oviedo. Sequences were compared to those in the databases with the BLAST (Basic Local Alignment Search Tool) program.

Plasmid DNA from clone *iviX* was recovered from the *Y. ruckeri* chromosome by triparental mating (11), and the DNA fragment situated upstream of the *cat* and *bla* genes was sequenced using the initial primer catseq-2 (5′-CGGTGGT ATATCCAGTG-3′), corresponding to nucleotides 31 to 15 of the *cat* gene from the pIVET8 plasmid (11).

***In vitro* regulation studies.** Cells of *Y. ruckeri* 150R*iviX* containing a *cdsB*::pIVET8 transcriptional fusion were incubated in the presence of different amino acids, such as L-serine, L-leucine, L-threonine, L-methionine, L-tryptophan, L-tyrosine, L-cysteine, and L-cystine, at concentrations of 0.5 mM and 20 mM in screw-top airtight tubes and flasks containing M9GC medium supplemented with 100 μg/ml ampicillin up to an OD_{600} of about 1.0. Cells were centrifuged at 12,000 × *g* for 5 min, and β-galactosidase activity was assayed in cells by the Miller method (23) using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. For the analysis of the maximal L-cysteine concentration required for induction of the *cdsAB* operon, L-cysteine was included in the culture medium in concentrations of 10 μM, 25 μM, 50 μM, 100 μM, 250 μM, 0.5 mM, 1 mM, 2 mM, and 5 mM, and β-galactosidase activity was assayed when culture reached an OD_{600} of 1.0. For time course induction experiments, 0.5 mM L-cysteine was added to the culture medium, and at different times of incubation β-galactosidase activity was determined.

Construction of *cdsA* and *cdsB* mutants. Internal fragments of 525 and 529 bp of the predicted *cdsA* and *cdsB* open reading frames (ORFs), respectively, were amplified by PCR with the following primers: forward primer cdsA-1 (5′-ATG C*GGATCC***TATTAGGCGCACTTTAT**-3′), with nucleotides 347 to 363 of the *cdsA* gene in bold type, and reverse primer cdsA-2 (5′-ATGC*GGATCC***TTAAC AGCTTCATCGTG**-3′), with nucleotides 881 to 865 in bold type, to amplify the internal fragment of the *cdsA* gene; forward primer cdsB-1 (5′-ATGC*AGATCT* **CGATACAGGAGAGTGAT**-3′), with nucleotides 356 to 372 in bold type, and reverse primer cdsB-2 (5′-ATGC*AGATCT***AGATATTCGGCCACCAC**-3′), with nucleotides 884 to 868 in bold type, to amplify the internal fragment of *cdsB*. Primers contained restriction sites for BamHI and BglII, in italics, and four additional bases at their 5′ end. The amplicons generated were digested with the corresponding enzymes and ligated into pJP5603 previously digested with BamHI and dephosphorylated. The ligation mixture was used to transform electrocompetent cells of *E. coli* S17-1λ*pir*. Selected transformants, containing the plasmid with the insert, were used to conjugate with *Y. ruckeri* 150R to obtain the *cdsA*::pJP5603 and *cdsB*::pJP5603 mutants, as previously described (14). The mutations were confirmed by Southern blot analysis after digestion of genomic DNA with EcoRI of the parental strain and the *cdsA* and *cdsB* mutants. The previously amplified internal fragments from *cdsA* and *cdsB* were used as probes. Probe labeling, hybridization, and developing were performed with the DIG DNA labeling and detection kit from Roche by following the manufacturer's instructions. The stability of the mutants in the absence of kanamycin was analyzed by doing several passes on nonselective medium, followed by comparison of the number of cells able to grow on plates with or without antibiotic.

To complete the sequence of *cdsB*, genomic DNA from the *Y. ruckeri cdsB* mutant was digested with EcoRI, the only restriction site on the pJP5603 plasmid. The restriction fragments were religated, and the mixture was used to transform cells of *E. coli* S17-1λ*pir*. Transformants were selected on 2× TY agar medium containing kanamycin. The plasmid containing the *cdsB* gene was sequenced with the initial primer RP (5′-CAGGAAACAGCTATGAC-3′) from the *lacZ* gene from the pJP5603 plasmid.

Growth studies and L-cysteine quantification. To check if *cdsA* and *cdsB* mutations played any role in the growth of *Y. ruckeri*, wild-type and mutant

strains were incubated in 50-ml Falcon screw-top airtight tubes containing M9GC and M9GC supplemented with 0.5 mM L-cysteine. Growth was monitored by determining the OD_{600} at different times during incubation at 18°C. Growth curves were determined in triplicate and repeated three times. For L-cysteine quantification in the supernatant of cultures at different incubation times through the growth curve, the acid ninhydrin assay described by Gaitonde (16) was used. Briefly, the content of this amino acid was determined colorimetrically by monitoring the absorbance at 560 nm and reading from a standard curve ($r^2 = 0.99$). M9GC supplemented with 0.5 mM L-cysteine with no cells was used as the control of the total amount of the amino acid in the medium. The experiment was repeated three times, and the mean and standard deviation were computed. Cystine (0.5 mM) was used as a negative control.

Amino acid transport assay. *Y. ruckeri* parental and *cdsA* strains were cultured in 50-ml Falcon screw-top airtight tubes containing M9GC medium supplemented with 0.5 mM L-cysteine at 18°C up to an OD_{600} of 0.8. Thirty milliliters of each strain was harvested, washed twice with buffer A, containing 100 mM Tris-HCl (pH 7.3), 100 mM NaCl, and 0.5 mM $MgCl_2$, and resuspended in 9 ml of the same buffer. After 5 min in the presence of chloramphenicol (15 μg/ml) to inhibit protein synthesis, a solution containing 2 mM L-cysteine as a carrier, 20 μCi/ml of ^{35}S-labeled L-cysteine (specific activity, 1,075 Ci/mmol) (Perkin Elmer, Boston, MA), and 10 mM dithiothreitol was added (final concentrations of L-cysteine and dithiothreitol were 200 μM and 1 mM, respectively). Assays were performed at 18°C, and duplicated samples of 0.5 ml were withdrawn at intervals, diluted 20-fold in cold buffer A, and filtered through 25-mm-diameter GF/C Millipore nitrocellulose membrane filters (0.45-μm pore size). Filters were washed with 10 ml of buffer A, dried, and immersed in Filter-Count scintillation cocktail (Perkin Elmer, Boston, MA), and the radioactivity was measured in a liquid scintillation counter. L-Cysteine uptake activity was calculated in nmol/min/mg protein. Total protein was quantified by the Bradford (AppliChem, Germany) protein method.

For competition studies, the different amino acids were added in 10-fold excess in relation to cysteine, except cystine, which was insoluble in buffer A at this concentration, and a 1:1 ratio was used. The rate of L-cysteine uptake was measured in a 5-min reaction. The proton gradient inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the cells 10 min before the labeled compound.

Experimental animal studies. To determine the role in virulence of *cdsA* and *cdsB* mutations, 50% lethal dose (LD_{50}) experiments with the parental and the mutant strains were carried out as described by Fernández et al. (14). Rainbow trout (*Oncorhynchus mykiss*) weighing between 8 and 10 g were kept in 60-liter tanks at 18 ± 1°C in continually flowing dechlorinated water. Groups of 10 fish were challenged by intraperitoneal injection of 0.1 ml serial 10-fold dilutions of an exponential-phase culture of bacteria in phosphate-buffered saline (PBS) over a range of 10^2 to 10^8 cells, and the mortalities were followed up for 7 days. Control fishes were injected with 0.1 ml PBS. Two different experiments were carried out, and the LD_{50} determinations were calculated by the method of Reed and Muench (28).

For *in vivo* competition assays, parental and mutant strains were grown separately in NB at 18°C in orbital shakers at 250 rpm up to an OD_{600} of 0.5 (approximately 10^8 cells/ml). Two and a half milliliters of parental and mutant strains were mixed, and 10-fold dilutions of this suspension were plated onto NA (to measure total CFU) and NA containing kanamycin (to determine mutant CFU). From this, the exact input ratio of mutant to wild-type CFU was calculated. A sample of 0.1 ml of the 10^{-2} dilution of both mixes (parental/*cdsA* or parental/*cdsB* mutant strains; approximately 10^6 cells/ml of each strain) was used to infect rainbow trout weighing from 8 to 10 g by intraperitoneal injection. After 72 h, spleen, liver, and intestine from each infected fish were homogenized together in NB with a stomacher. Aliquots of the suspensions were plated onto NA and NA containing kanamycin as a selective medium to determine the output ratio of mutant to parental cells. In each experiment, three fish were used, and the competence index (CI) was determined as the mean of data of the three fish. The experiment was repeated twice, and the mean and standard deviation were determined. The competence index is defined as the output ratio (mutant/parental) divided by the input ratio (mutant/parental). Animal experiments were performed in accordance with the European legislation governing animal welfare, and they were authorized and supervised by the Animal Experimentation Ethics Committee of Universidad de Oviedo.

PCR detection of *cdsA* and *cdsB* in different *Y. ruckeri* strains. Fifteen *Y. ruckeri* strains were studied in regard to the presence of *cdsA* and *cdsB* genes. PCR was performed using the following primers: DetC-1 (5′-ATGCGGATCCTTTCATT ATGTTAGTTA-3′) and DetC-2 (5′-CCATTATTCCATTTTGA-3′) to check the presence of the *cdsA* gene, and cdsB-1 and cdsB-2 (described previously) to check the presence of the *cdsB* gene. All PCR components (DNA polymerase, reaction buffer, and deoxynucleoside triphosphates) were provided by Biotools. The amplification reactions were performed in a Perkin-Elmer 9700 GeneAmp thermocycler with an initial denaturation cycle at 94°C for 5 min, followed by 25 cycles of amplification (denaturation at 94°C for 30 s, annealing at 38°C for *cdsA* and 44°C for *cdsB* for 60 s, and extension at 72°C for 1 min), and a final 7-min elongation step at 72°C. The reaction products corresponding to the two groups were mixed, and 1.5% agarose gel electrophoresis was used to separate the generated PCR amplicons.

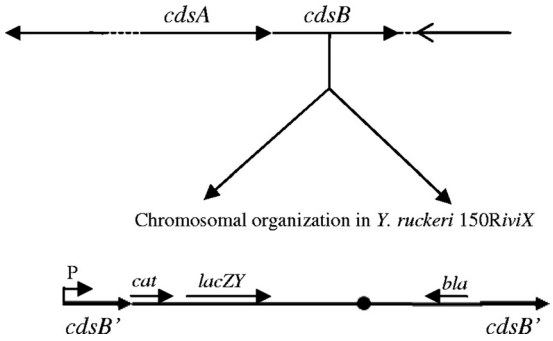

FIG. 1. Chromosomal arrangement of the region containing *cdsA* and *cdsB* genes in *Y. ruckeri* 150R. Arrows indicate the direction of the transcription. The organization of the transcriptional fusion between *cdsB* and the promoterless genes *cat* and *lacZY* in *Y. ruckeri* 150R*iviX* is shown underneath, and the putative promoter (P) selected by IVET is indicated. Catseq-2 oligonucleotide was used to sequence the fragments adjacent to the pIVET8 integration site. *cat*, chloramphenicol acetyltransferase gene (promoterless); *lacZY*, genes for lactose fermentation (promoterless); *bla*, ampicillin resistance gene.

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the sequences reported in this paper is HM769941.

RESULTS

CdsB shows conserved amino acid positions with respect to L-cysteine desulfidase enzyme. Selection of specific *in vivo*-induced genes in *Y. ruckeri* was previously carried out by IVET (11). One out of 14 isolated clones, the *iviX*, contained an *in vivo*-induced transcriptional fusion (Fig. 1) with a partial ORF encoding a protein which showed a significant similarity with hypothetical transmembrane proteins from different bacteria (11). Further sequencing and analysis of the adjacent region of this genetic locus revealed the presence of an additional ORF separated by 49 bp and proceeded by a consensus −10 (TT TAAT) and −35 (CAGATA) promoter sequence and a ribosome binding site (RBS) (AGGCGA). The upstream gene consists of 1,326 bp and encodes a protein of 442 amino acids which shares a high identity with hypothetical proteins of *Y. ruckeri* ATCC 29473 (442/442 [100%]), *Y. enterocolitica* (418/442 [94%]), *Salmonella enterica* serovar Typhimurium LT2 (389/445 [87%]), and *Citrobacter koseri* (387/445 [86%]) and with the protein YhaO of *Escherichia coli* B (162/175 [92%]). The translational compound encoded by the first ORF shows 11 transmembrane helixes (putative membrane localization) and a conserved domain related to transmembrane amino acid transporter proteins. The second ORF of 1,323 bp encodes a protein of 441 amino acids which shares high identity with hypothetical proteins of *Y. ruckeri* ATCC 29473 (441/441 [100%]), *Y. enterocolitica* (329/439 [74%]), *Escherichia albertii* TWO7627 (300/440 [68%]), and *Salmonella enterica* CT-02021853 (296/440 [67%]) and the protein YhaM CFTO73 of *Escherichia coli* B (297/441 [67%]). It encodes a protein

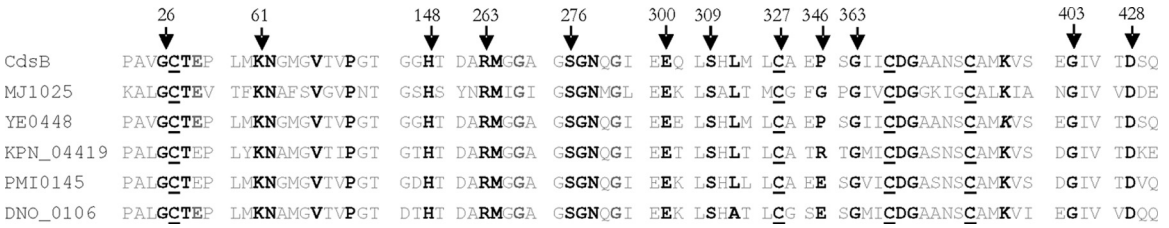

FIG. 2. Conserved residues among the orthologs of CdsB. GenBank data accession numbers correspond to *M. jannaschii* (MJ1025), *Y. enterocolitica* (YE0448), *K. pneumoniae* (KPN_04419), *P. mirabilis* (PMI0145), and *D. nodosus* (DNO_0106). Note that the amino acid variations correspond with position 346 of the CdsB protein. An additional variation is found in the 311 position of *D. nodosus* (DNO_0106). The four cysteines C27, C327, C366, and C373 described by Tchong et al. (33) as possible ligands for the [3Fe-4S] center are underlined.

which presents two conserved domains: COG3681 (L-cysteine desulfidase) and cI12120 (L-serine dehydratase, iron-sulfur-dependent, alpha subunit). Similar domains exist in the L-cysteine desulfidase of *M. jannaschii*, which catalyzes the breakdown of L-cysteine into pyruvate, ammonia, and sulfide (33). In fact, the protein encoded by the second ORF in *Y. ruckeri* presents 28 out of 29 amino acids conserved in specific positions of the L-cysteine desulfidase of *M. jannaschii*, including the four cysteines (C27, C327, C366, and C373) described by Tchong et al. as possible ligands for the [3Fe-4S] center (33) (Fig. 2). The differential amino acid position corresponds to P346 in *Y. ruckeri*, and it also differs from *M. jannaschii* in other orthologous loci found in *Y. enterocolitica*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, among others (Fig. 2). On the basis of these amino acid identities and domains as well as other results shown below, from now on the two ORFs identified from the analysis of the *iviX* clone are designated *cdsB* (cysteine desulfidase) and *cdsA*, for ORF2 and ORF1, respectively.

***cdsA* and *cdsB* orthologs are present in several bacterial groups.** Comparative nucleotide sequence analysis using the BLAST program showed that similar *cdsAB* clusters were present in *C. koseri* ATCC BAA-895 (CKO_04511 and CKO_04510), *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 (*yhaO* and *yhaN*), *Shigella sonnei* Ss046 (*yhaO* and *yhaNM*), *Escherichia coli* B strain REL606 (*yhaO* and *yhaM*), *Dichelobacter nodosus* VCS1703A (DNO_0107 and DNO_0106), *Chromobacterium violaceum* ATCC 12472 (CV_1823 and CV_1824), and *Y. enterocolitica* subsp. *enterocolitica* 8081 (YE0447 and YE0448) (Fig. 3). However, the cluster was absent from species closely related to *Y. ruckeri* and *Y. enterocolitica*, such as *Y. pseudotuberculosis* IP 32953 and *Y. pestis* CO92. These two species present similar *cdsA* loci (YPTB0334

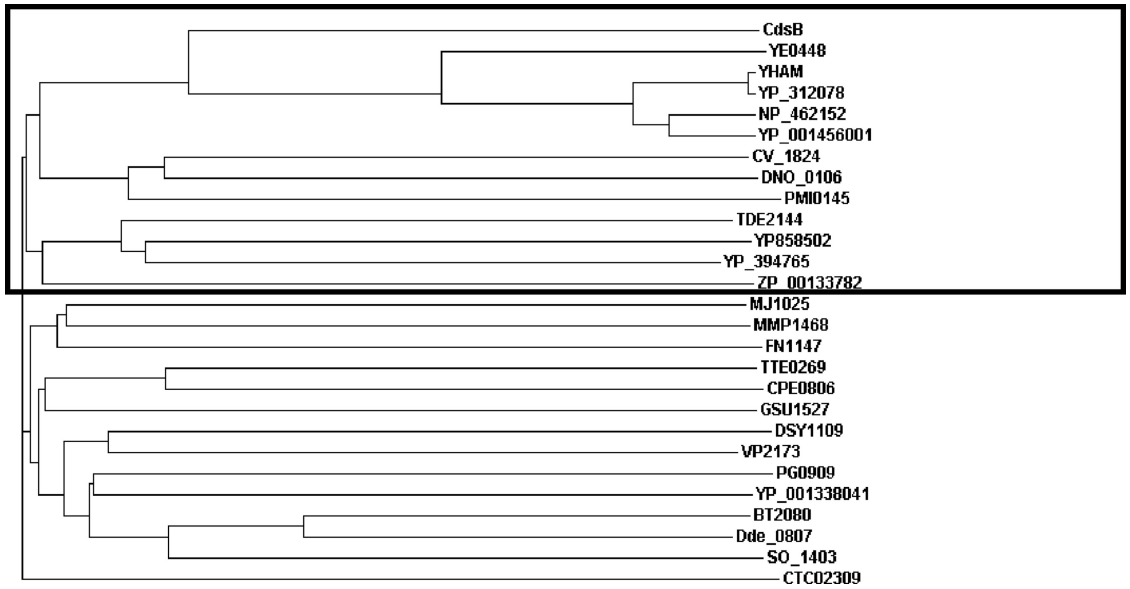

FIG. 3. Phylogenetic tree based on the CdsB protein sequence, showing the relationships between *Y. ruckeri* and its other relatives. GenBank data accession numbers are indicated: *Y. enterocolitica* (YE0448), *Escherichia coli* (YHAM), *Shigella sonnei* Ss046 (YP_312078), *Salmonella enterica* (NP_462152), *Citrobacter koseri* (YP_001456001), *Chromobacterium violaceum* (CV_1824), *Dichelobacter nodosus* (DNO_0106), *Proteus mirabilis* (PMI0145), *Treponema denticola* (TDE2144), *Aeromonas hydrophila* (YP858502), *Lactobacillus sakei* (YP_394765), *Actinobacillus pleuropneumoniae* (ZP_00133782), *Methanocaldococcus jannaschii* (MJ1025), *Methanococcus maripaludis* (MMP1468), *Fusobacterium nucleatum* (FN1147), *Thermoanaerobacter tengcongensis* (TTE0269), *Clostridium perfringens* (CPE0806), *Geobacter sulfurreducens* (GSU1527), *Desulfitobacterium hafniense* (DSY1109), *Vibrio parahaemolyticus* (VP2173), *Porphyromonas gingivalis* (PG0909), *Klebsiella pneumoniae* (YP_001338041), *Bacteroides thetaiotaomicron* (BT2080), *Desulfovibrio desulfuricans* (Dde_0807), *Shewanella oneidensis* (SO_1403), and *Clostridium tetani* (CTC02309). Note that the species grouped in the first division from CdsB of *Y. ruckeri* to DNO_0106 of *Dichelobacter nodosus* presented a *cdsAB* orthologous operon in their genome. The large black box groups the species close to *Y. ruckeri* which harbor the *cdsAB* cluster.

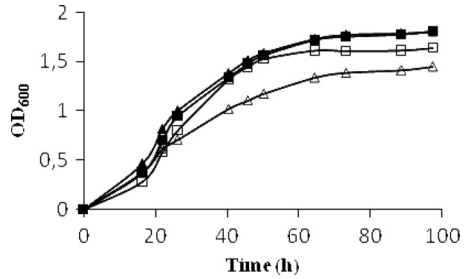

FIG. 4. L-Cysteine effect on the growth of *Y. ruckeri* parental and *cdsA* mutant strains. Strains were grown under simulated anaerobic conditions in M9GC (black symbols) and M9GC supplemented with 0.5 mM L-cysteine (white symbols). ▲ and △, parental strain; ■ and □, *cdsA* mutant. Growth curves were determined by three independent experiments, and the media were represented. The standard deviations were lower than ±0.06.

and YPO0277, respectively) in their genomes, but these are followed by the *metC* gene, which encodes a cystathionine β-lyase. Interestingly, this enzyme also catalyzes the degradation of L-cysteine to pyruvate, ammonia, and sulfide in *Escherichia coli* (3). A second group of bacteria, including *M. jannaschii*, was found in which the *cdsB* locus was not preceded by the corresponding *cdsA* locus. A phylogenetic analysis based on CdsB protein sequence showed the relationship between *Y. ruckeri* and its other relatives (Fig. 3).

The *cdsAB* operon is induced specifically by L-cysteine. The strain *Y. ruckeri* 150R*viX*, obtained by IVET, contains a transcriptional fusion between the *cdsAB* promoter and *lacZY* (Fig. 1). This construction was used for the analysis of the expression of these genes in response to different culture conditions. The results obtained by β-galactosidase activity determination in cultures grown in 50-ml Falcon screw-top airtight tubes containing M9GC medium showed that L-cysteine and L-cystine were the only promoter inductors. Specifically, β-galactosidase activity was increased approximately 4-fold in the presence of 0.5 mM concentrations of these amino acids. This increase was detected in both exponential- and stationary-phase cultures. The catabolism of these amino acids produced the generation of abundant H_2S, as was determined by its characteristic unpleasant odor and the production of a black precipitate after culture centrifugation when an iron salt was included in the culture medium. It should be noticed that during incubation under these conditions and in the presence of a reducing agent such as H_2S, which changed the redox potential of cultures, L-cystine turned into L-cysteine, as was determined by the ninhydrin method (data not shown). This, together with the limited induction (1.7-fold) observed in the presence of L-cysteine when incubation was carried out under oxygenation conditions, in which L-cysteine is turned into L-cystine, strongly suggests that L-cysteine is the unique operon inductor, and a progressive increase in β-galactosidase activity was found until a maximum at a 1 mM concentration of the amino acid. Other amino acids, such as leucine, serine, threonine, tryptophan, tyrosine, and methionine, in both low (0.5 mM) and high (20 mM) concentrations, did not significantly induce the promoter.

The *cdsA* mutant but not the *cdsB* mutant is more resistant to L-cysteine than the parental strain due to an alteration in L-cysteine uptake. To investigate the relative roles of the *cdsAB* operon in bacterial physiology, two mutants with independent mutations in *cdsA* and *cdsB* were constructed by insertional mutagenesis as described by Fernández et al. (11). PCR and Southern blot analysis showed that the genes *cdsA* and *cdsB* were inactivated in the respective mutants, and both were genetically stable under nonselective antibiotic pressure (data not shown). No differences in growth were detected between the parental and the mutant strains (*cdsA* and *cdsB* mutants) when they were grown in screw-top airtight tubes containing M9GC or in M9GC supplemented with specific amino acids, such as threonine, serine, tryptophan, or tyrosine. A similar result was obtained when the complex medium NB was used. However, the parental and the *cdsB* mutant strains showed a significant growth limitation in medium containing 0.5 mM cysteine, while the *cdsA* mutant partially overcame this growth limitation (Fig. 4).

To determine if the partial resistance of the *cdsA* mutant to cysteine was a consequence of a limited consuming capacity of this amino acid, the cysteine present in the medium was quantified during the growth curves of *Y. ruckeri* parental and mutant strains in M9GC medium plus 0.5 mM cysteine. As can be observed in Table 2, the cysteine remaining in the culture medium after 48 h of incubation was considerably higher in the *cdsA* mutant than in the parental and *cdsB* mutant strains. This difference was observed along the whole growth curve. Therefore, the limited toxic effect that cysteine has in the growth of the *cdsA* mutant, together with its limited capacity for cysteine consumption, suggests that the *cdsA* gene encodes a cysteine permease.

In order to confirm the role of CdsA in cysteine uptake, studies using [^{35}S]L-cysteine were carried out. Cells of the *cdsA* mutant and parental strains were grown in the presence of 0.5 mM cysteine, and at mid-logarithmic-growth phase (OD_{600} = 0.7 to 0.8) cysteine uptake was measured. When the kinetics of L-cysteine uptake were determined in the presence of a 200 μM concentration of this amino acid, the results showed a significant delay in the uptake of radiolabeled cysteine in the *cdsA* mutant compared to that in the parental strain (Fig. 5).

To define the substrate specificity of CdsA, competitive transport studies were carried out in the presence of a 10-fold excess of unlabeled competing ligand. Among the various amino acids used, only L-methionine, L-cystine, and to a lesser extent L-glutamic acid compete with the cysteine uptake (Table 3). The other tested amino acids (tryptophan, leucine, serine,

TABLE 2. L-Cysteine remaining in the supernatant of cell cultures of *Y. ruckeri* parental and *cdsA* and *cdsB* mutant strains after 48 h of incubation in M9GC supplemented with 0.5 mM L-cysteine

Strain	L-Cysteine in culture supernatant (mg/ml)	Utilization of L-cysteine (mg/mg protein)[a]
No cells	0.065 ± 0.001	
Y. ruckeri parental	0.005 ± 0.002	1.02 ± 0.09
Y. ruckeri cdsA mutant	0.038 ± 0.001	0.27 ± 0.08
Y. ruckeri cdsB mutant	0.006 ± 0.003	0.89 ± 0.08

[a] The utilization of L-cysteine was inferred per milligram of protein, considering the amount of this amino acid present at the end of the incubation period in the culture medium without cells. The data represent the average of results from three independent experiments.

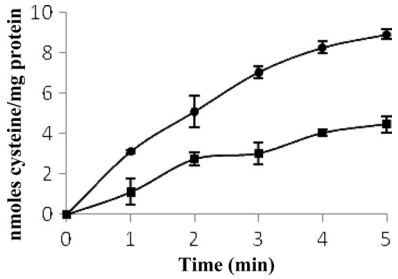

FIG. 5. Uptake of [^{35}S]L-cysteine by *Y. ruckeri* parental and *cdsA* mutant strains. L-Cysteine taken up by cells of parental (●) and *cdsA* mutant (■) strains during a 5-min period. The data points represent the mean values from three separate experiments.

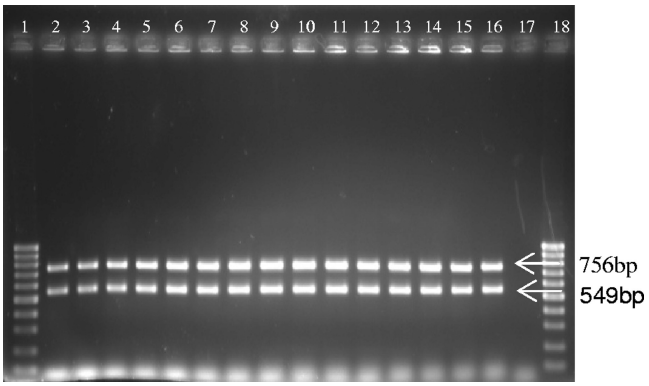

FIG. 6. PCR detection of *cdsA* and *cdsB* genes from different *Y. ruckeri* strains. Independent PCRs were carried out for each gene. The amplicons obtained were then mixed and separated in a 1.5% agarose gel. The sizes of the amplicons generated were 756 bp and 549 bp for *cdsA* and *cdsB*, respectively. Lanes: 2, strain 146; 3, strain 147; 4, strain 148; 5, strain 149; 6, strain 150; 7, strain 955; 8, strain 956; 9, strain 35/85; 10, strain 13/86; 11, strain 43/19; 12, strain A100; 13, strain A102; 14, strain 150/05; 15, strain 158/05; 16, strain 382/05; 17, negative control. Lanes 1 and 18, DNA molecular size markers from 1,000 to 100 bp.

and threonine) had no effect on the cysteine uptake. The presence of the uncoupler of phosphorylation CCCP resulted in a considerable loss in the cysteine uptake, which implies that CdsA-mediated cysteine transport is an energy-dependent process (Table 3).

The *cdsAB* operon is needed for full virulence of *Y. ruckeri*, and it is conserved in different isolates. The fact that the *Y. ruckeri* 150R*iviX* strain was obtained as an IVET clone suggested that the *cdsAB* operon could have a role during the infection process. To go further into this, *in vivo* competition assays and LD_{50} experiments were carried out using the parental and the *cdsA* and *cdsB* strains. The competence indexes (CI) obtained for *in vivo* experiments were 0.03 ± 0.01 and 0.24 ± 0.14 for the *cdsA* and *cdsB* mutants, respectively, which indicated a significant lower recovery of cells of the mutant strains than that of the parental strain. The results obtained in LD_{50} experiments showed that the mutant strains showed significantly higher values than those of the parental strain. Thus, the means of LD_{50} values were 5.65×10^4 CFU/fish for the parental strain and 1.18×10^6 and 3.55×10^5 CFU/fish for the *cdsA* and *cdsB* mutants, respectively. Therefore, gene IVET selection, together with the results obtained from *in vivo* CI and LD_{50} experiments, confirmed that the *cdsAB* operon is necessary for full virulence and thus for the development of the infection process.

TABLE 3. Effect of different compounds on L-cysteine transport by cells of *Y. ruckeri*

Compound	% uptake ± SD
No inhibitor	100
L-Cysteine	43 ± 12
L-Threonine	98 ± 9
L-Serine	109 ± 3
L-Leucine	75 ± 16
L-Methionine	39 ± 10
L-Tryptophan	86 ± 8
L-Glutamic acid	63 ± 14
L-Cystine[a]	51 ± 14
CCCP[b]	36 ± 1
DMSO	95 ± 12

[a] [^{35}S]L-cysteine was used at a concentration of 200 μM. All competitors were added to a final concentration of 2 mM, a 10-fold excess over the labeled substrate, except L-cystine, added in a final concentration of 200 μM.

[b] Cells were preincubated with the metabolic inhibitor CCCP for 10 min before addition of the [^{35}S]L-cysteine. CCCP was dissolved in dimethyl sulfoxide (DMSO), and hence this compound was analyzed for its effect on L-cysteine uptake. The data represent the mean values from three independent experiments. When [^{35}S]L-cysteine was used at a concentration of 20 μM and all the competitors were added to a final concentration of 200 μM, the results were similar.

The presence of the *cdsAB* operon was analyzed by PCR in 15 different *Y. ruckeri* strains from different sources and origins. All of them showed the amplification of two bands of 756 bp and 549 bp, corresponding to internal fragments of *cdsA* and *cdsB*, respectively (Fig. 6). This result indicates intraspecies genetic homogeneity, given that the genes are present in serotype I and II as well as recently isolated new biotype strains from outbreaks in Spain.

DISCUSSION

Once more, IVET has allowed the in-depth investigation of the pathogenic mechanisms of *Y. ruckeri*. In this case, analysis of the sequences surrounding the *iviX* clone (11) revealed the presence of two ORFs. Comparative analysis and functional predictions indicated that they could represent a new system involved in the uptake and further degradation of the amino acid cysteine. Major clues were the presence in the CdsB protein of 28 out of 29 conserved amino acids of the L-cysteine desulfidase of *M. jannaschii* (33) and the presence in CdsA of a clear permease domain related to amino acid transporter proteins.

In the present work, it is shown that L-cysteine desulfidase orthologs were present in many facultative bacteria apart from *E. coli* (33), such as *C. koseri*, *Y. ruckeri*, *K. pneumoniae*, and *Y. enterocolitica.* Interestingly, unlike the L-cysteine desulfidase of *M. jannaschii*, the CdsB protein of these species is preceded in the genome by the permease CdsA. In fact, phylogenetic analysis based on the CdsB protein showed that these bacteria and others which also harbor the *cdsAB* loci are grouped in the same cluster, suggesting a common evolutionary origin. In other species, such as *Y. pestis* and *Y. pseudotuberculosis*, downstream from a locus similar to *cdsA* is located *metC*, a gene

involved in the degradation of L-cysteine to pyruvate, ammonia, and sulfide in *E. coli* (3). The fact that *cdsA* appears adjacent to two different enzymes which use L-cysteine as a substrate reinforces the role of this protein as a cysteine permease.

The relation of these two genes with the uptake and further metabolism of L-cysteine was also supported by promoter induction experiments, comparative toxic effect, and cysteine consumption throughout the growth of parental and *cdsA* mutant strains and also by the differential cell uptake of labeled cysteine observed between the two strains.

In fact, promoter induction took place only in the presence of cysteine. The induction detected when cystine was in the culture medium seems to be a consequence of the conversion of this amino acid to cysteine. This was supported by the ninhydrin method, which detected high levels of cysteine in culture supernatants when cystine was the only amino acid included in the medium. The production of H_2S as a metabolite derived from cysteine and cystine utilization helped to keep a reductive state, as was confirmed by measuring the redox potential. On the contrary, under aerobic conditions, only limited induction was observed, probably due to the rapid conversion of cysteine to cystine (10). The fact that in aerobic conditions neither cysteine nor cystine produced significant promoter induction strongly supports the idea that this operon is induced specifically by cysteine. It should be emphasized that the genes are found widely in anaerobic and facultative bacteria but not in aerobic bacteria.

The analysis of mutations in *cdsA* and *cdsB* genes also supported the involvement of the *cdsA* gene in the uptake of cysteine. The cysteine toxic effect defined by Kari et al. (18) was lower in the *cdsA* mutant strain than in the parental and *csdB* mutant strains, probably due to the lesser utilization of cysteine by the *cdsA* mutant in the course of growth, just as was shown by the ninhydrin method.

Finally, radiotracer studies confirmed the involvement of *cdsA* in the uptake of L-cysteine in *Y. ruckeri*. Time course results as well as L-cysteine consumption during the growth curve suggested the existence of at least one additional cysteine uptake system given the residual cysteine uptake in the *cdsA* mutant. However, the comparative analyses of kinetic data in different biological systems are complex and subject to multiple factors. These, together with the special characteristics of this amino acid (in oxidizing conditions, cystine is the compound present in the environment), make extremely difficult the estimate and comparison of specific kinetic parameters between strains. These are the reasons information concerning cysteine uptake in bacteria is sparse and few examples of cysteine transporters have been found. In *Campylobacter jejuni*, CjaA is a receptor protein with a high affinity for L-cysteine (24). In *Legionella pneumophila* as well as in *Cyanobacterium synechocystis*, two cysteine transporters were identified (10, 32). In *Saccharomyces cerevisiae*, up to seven different permeases were described as taking up cysteine, and the uptake of this amino acid was found to be a nonsaturable process under various conditions (9).

The presence of cysteine desulfhydrases has been described in different microorganisms. In *Bacillus subtilis*, four different genes encoding this kind of enzyme have been found (2). Interestingly, in *E. coli*, a quintet mutant strain in five different cysteine desulfhydrases still showed some cysteine desulfhydrase activity (4). According to all the data, it seems that the *cdsAB* system remained undiscovered because specific culture conditions are needed for induction. It could represent one of the systems responsible for the residual cysteine degradation activity detected in this *E. coli* quintet mutant strain, given that *E. coli* possesses an orthologous *cdsAB* operon.

It should be indicated that local reducing environments caused by H_2S generation could be present in the gut tract of rainbow trout, one of the main locations of *Y. ruckeri* (14), as a consequence of bacterial metabolism. H_2S is also present in other tissues (25), since this gas is considered to be a gasotransmitter able to trigger cell signaling in vertebrate animals, including rainbow trout, with physiological levels of H_2S in the blood in the range from 10 to 300 μM (8, 21). It is possible that H_2S reduces cystine to cysteine under these conditions, and genes related to the transport and metabolism of this amino acid might be induced. This CdsAB system might be involved in the generation of iron-sulfur centers for proteins, as was proposed for the L-cysteine desulfidase of *M. jannaschii* (33), and also in glutathione accumulation, the major redox buffer and detoxification molecule in the cell. In any case, the absence of the CdsA and CdsB proteins led to bacterial attenuation as well as a significantly restricting growth in the fish, showing that this system is related to the progression of the bacteria inside the animal during the infection process. According to the presence of the *cdsAB* operon in different pathogenic species, it is probable that its involvement in the infection process is not exclusive to *Y. ruckeri.*

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